Eur J Plant Pathol (2011) 131:661–668
DOI 10.1007/s10658-011-9840-0

Neofusicoccum parvum associated with fruit rot and shoot blight of peaches in Greece

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Accepted: 11 July 2011 / Published online: 26 July 2011

Abstract Shoot blights and fruit rots comprise the most serious diseases of peaches in Greece. In this study, the importance of the fungus *Neofusicoccum parvum* as a casual agent of a fruit rot and shoot blight of peach trees in Greece was investigated. This pathogen was isolated from both immature and mature peach fruit of the cultivar "Catherine" and later on from mature fruit of the peach cultivars "Andross", "RedHaven", "Sun Crest" and "Sun Cloud". In the first year of investigation, *N. parvum* was found causing preharvest fruit rot and shoot blights of peach trees only at the location "Ammos-Mesi-Meliki Verias" in the prefecture of Imathia (the main peach production area of Greece) at incidences of 30 and 8%, respectively. However, in 2006 *N. parvum* was isolated from more locations such as Diavatos, Veria, Kopanos and Agia Marina in the prefecture of Imathia, but only at less than 3% of the total surveyed rotted peach fruit and blighted shoots. The pathogen overwintered as sub-epidermal pycnidia in blighted shoots or mummified fruit that remained on peach trees. This study also showed that the optimum temperature for mycelial growth and conidial germination of *N. parvum* was 25ºC. Pathogenicity tests using peach fruit showed that isolates of *N. parvum* and *Diplodia seriata* (isolated from pistachio grown in the same region) showed no significant differences in their virulence. In laboratory inoculation tests using detached shoots from 25 peach and nectarine cultivars, *N. parvum* isolates obtained from rotted peaches caused different size cankers on these cultivars. The cultivar Big Top was the most susceptible while the cultivar Maria Bianca the least susceptible.

Keywords *Neofusicoccum parvum* · Fruit rot · Pathogenicity · Peaches · Shoot blight · Virulence

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Introduction

Imathia County is the main fresh and canned peach production area of Greece. Preharvest fruit rots and shoot blights constitute serious problems in cultivation of peach trees in this area due to humid environment. Brown rot, caused by *Monilinia laxa*, is considered to be the main cause of fruit rots and shoot blights on peaches in Greece. Some other pathogens such as *Colletotrichum* spp., *Fusarium* spp., *Botrytis cinerea, Rhizopus stolonifer, Mucor piriformis* and *Aspergillus niger* have been isolated from rotted peaches, while the pathogens *Colletotrichum* spp., *Fusicoccum* sp., and *Cytospora* sp. have also been

reported as causal agent of shoot blights in Greece (Pantidou 1973).

The fungus *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, previously known as *Botryosphaeria parva*, has been reported as a pathogen of pome and stone fruit trees (Slippers et al. 2007). In addition, this pathogen has been isolated from many other hosts, such as blueberry (Espinoza et al. 2009), *Syzygium cordatum* (Pavlic et al. 2009), avocado (McDonald et al. 2009), grapevine (Laveau et al. 2009), eucalyptus (Slippers et al. 2009), and walnut. Other species of Botryosphaeriaceae such as *Botryosphaeria dothidea* are associated with fruit rots and fungal gummosis of peach trees in many countries (Cedelo et al. 1994; Combrink et al. 1984; Caponero et al. 1999, 2001; Beckman et al. 2003). Although *N. parvum* has been associated with serious problems of peach cultivation in other contries, there has been no investigation of the importance of this pathogen in Greece.

The use of resistant cultivars is important in controlling diseases because growers do not spend funds for extraneous control measures such as fungicides. Cultivar resistance can be a useful complementary control measure even when fungicides must be used. Differences in the susceptibility of peach and nectarine cultivars on diseases caused by *B. dothidea* have been reported (Beckman and Reilly 2005). In addition, previous works showed differences in virulence and genetic variation among isolates of *B. dothidea* originated from different hosts (Ma and Michailides 2002; Ma et al. 2001).

The objectives of this study were to: a) investigate the distribution of *N. parvum* in Imathia County, the main peach production area of Greece, in two consecutive years (2005 and 2006); b) test the pathogenicity of two *N. parvum* isolates originated from rotted peaches on 24 peach and nectarine cultivars; and c) compare the virulence of *N. parvum* with that of four *D. seriata* isolates.

Materials and methods

Isolation and identification of *Neofusicoccum parvum*

In June 2005, immature peach fruits of the cultivar "Catherine" with an unusual rot were collected from a peach orchard located in Mesi, Verias, and transferred to the laboratory of the Pomology Institute, in Naoussa. Isolation of the pathogen was made by cutting 3×3×3 mm pieces of internal fruit tissues and plating on potato dextrose agar (PDA) acidified (2.5 ml 85% lactic acid/l). A fast growing fungal species was consistently isolated. Identification of the pathogen was based on morphological characteristics and confirmed (two isolates) based on ITS and EF sequence by the CBS Fungal Biodiversity Centre, Identification Service (Utrecht, Netherlands).

Distribution of *N. parvum* in Imathia

To investigate the distribution of *Neofusicoccum parvum* in the Prefecture of Imathia, Greece, 300 rotted fruit and 200 blighted shoots each of the peach cultivars "Catherine", "Andross", "RedHaven", "Sun Crest" and "Sun Cloud" were collected from various commercial orchards and transferred to the laboratory for isolation of putative pathogens in both 2005 and 2006. Again, isolation of the causal pathogens was done as described above and the percentage of rotted fruit and blighted shoots by *Neofusicoccum parvum* was recorded.

To investigate the overwintering structures of *Neofusicoccum parvum*, 200 blighted shoots and 200 mummies from peach orchards were examined microscopically in late autumn and winter. When necessary, isolations on acidified potato dextrose agar were made to confirm the fungus.

Effect of temperatures on mycelial growth and conidial germination of *N. parvum*

Mycelia growth An agar disk, 6 mm in diameter, taken from an active colony of *N. parvum*, which originated from a rotted fruit of the cv. Catherine, was placed in the centre of each of five replicated dishes (9 cm diameter) containing PDA. Three single-spore isolates were used. The dishes were then incubated in a growth chamber at 2–4, 10, 15, 20, 25, 30, and 35°C for 2 days and the diameter of the resulting colony was recorded. This experiment was repeated.

Conidia germination Conidia production was made according to method described by Amponsah et al. (2008). The same three single spore isolates were used. To harvest the conidia from the PDA slants a solution of Tween 20 wetting agent (BDH Ltd., Poole, UK) was prepared by measuring 50 μl of Tween 20 with an micropipette and mixing this into 100 ml of

sterile distilled water in sterile glass beakers using a glass rod. Five ml of this solution was pipetted into one of the agar slants and the conidia agitated gently by rubbing with a flamed loop. To ensure a sufficient conidia density, the Tween/conidia suspension was tipped into a second agar slant and the process repeated and finally into a third slant and repeated. The resulting suspension was then filtered to remove any mycelium/agar fragments through 2 layers of moistened sterile cheesecloth placed in a small glass funnel resting in a 50-ml glass beaker.

The suspension was added to a 10 ml potato dexrose broth giving a final conidia density of 8.18 x 10^5/ml determined by using a Neubauer improved counting chamber/haemocytometer). The lids were fully tightened and each container shaken gently to thoroughly mix the suspension. The bottles were placed in a growth chamber at 2–4, 10, 15, 20, 25, 30, and 35°C for 12 h. To determine the percentage of conidial germination, 100 conidia were counted under the microscope at×400 by moving the stage whilst not attempting to randomise the observations. A conidium was considered germinated when the germ tube length was equal to the greatest diameter of the swollen conidium (1 to 1.5×). This experiment was conducted twice.

Pathogenicity and virulence of *N. parvum*

To reproduce symptoms in the laboratory, immature and mature fruits of the peach cultivar "Catherine" were disinfested by dipping in 10% sodium hypochlorite for 15 min and then were artificially inoculated. Inoculation was made by wounding (2×2 mm) the fruit with a sharp sterile nail and placing a 40-µl drop of a 1×10^4 spores/ml suspension prepared from a fresh culture of the fungus. Inoculated fruit were lightly enclosed in special plastic containers and the containers placed in a growth chamber at 24–26°C. Fruit treated similarly but using a drop of sterile-distilled water instead of the spore suspension served as control. Symptom development was recorded 7 days after inoculation and incubation of fruit and decay lesions were measured.

The assay using excised shoots described by Matheron and Mircetich (1985) was used to examine the pathogenicity of two *N. parvum* isolates (originating from a rotted peach fruit) on 24 peach and nectarine cultivars (Table 1). Twenty segments of woody shoots, 10 cm in length and 1.5 to 2 cm in diameter, were collected from each cultivar, and 10 each per isolate. A mycelial plug of 6 mm in diameter taken from the margins of a 4-day-old culture was inserted in the middle of each excised shoot piece under the bark. The plug and the wound were covered with petroleum jelly and wrapped with adhesive tape to prevent desiccation. Inoculated shoot segments were incubated at 25°C in moist chambers for 14 days after which the length of the resulting necrosis was recorded. This experiment was repeated twice.

Table 1 Susceptibility of peach and nectarine cultivars to *Neofusicoccum parvum* infection in Imathia, Greece

Cultivars	Species	S.E.	Length of Necrosis (mm)	
Big Top	Nectarine	5.354	31.10[x]	a
Sun Cloud	Peach	1.765	25.02	b
Sweet Gold	Peach	2.168	24.98	b
Symphony	Peach	1.762	24.91	bc
Fantasia	Nectarine	2.177	23.48	bcd
O'Henry	Peach	1.257	23.25	bcd
RedHaven	Peach	2.489	21.61	bcde
Fayette	Peach	2.161	21.44	bcde
Tasty Free	Nectarine	4.793	21.39	bcde
Fire Blight	Nectarine	1.479	21.10	bcde
Red Gold	Nectarine	1.040	21.02	bcde
Venus	Nectarine	1.082	19.47	cdef
June Gold	Peach	0.551	19.41	cdef
Early Gold	Peach	0.373	19.05	cdef
Sun Crest	Peach	0.789	18.64	def
Harmony	Peach	0.995	18.52	def
Crest Haven	Peach	0.715	18.51	def
First Gold	Peach	1.006	18.32	def
May Grand	Nectarine	0.596	18.07	ef
Andriana	Nectarine	0.701	18.03	ef
Caltesse 2000	Nectarine	0.792	17.88	ef
H.d. Hale	Peach	0.914	17.47	ef
Rita Star	Nectarine	0.306	17.45	ef
Maria Bianca	Peach	0.984	15.31	f

[x] Values are the means of two experiments with 2 isolates each; Values in the same column followed by different letters were significantly different at $P=0.05$ according to the Wald Test

Pathogenicity and virulence of *N. parvum* and *D. seriata* isolated from other hosts

The pathogenicity and virulence of four *D. seriata* isolates (two from pistachio trees one from persim-

mon, and one from walnut) and that of two *N. parvun* isolates from rotted peaches were compared. All the *D. seriata* isolates were identified by the CBS Fungal Biodiversity Centre, Identification Service (Utrecht, Netherlands). The isolates were cultured in Petri dishes containing PDA and incubated at 25°C for 5 days. For inoculation of peaches, a sterilized transfer needle was used to make a small wound on the surface of each fruit, and a 3-mm mycelial plug taken from the edge of a colony of each isolate was placed onto the wound. Two hundred forty peaches were used, 40 for each isolate. Forty peaches, inoculated with agar without mycelium, were used as controls. Peaches were then incubated at room temperatures (25±2ºC) for 5 days after which the diameter of decay lesion was recorded. This experiment was repeated once.

Statistical analyses

To analyse data for significant differences at $\alpha=0.05$, the Generalized Linear Model (Wald's Chi-Square) was applied (SPSS Grad Pack 16 for Windows).

Results

Isolation and identification of *N. Parvum*

A fungus of the Botryosphaeriaceae family was isolated from rotted immature fruit of the peach cultivar 'Catherine". At an early stage, before the appearance of pycnidia, the symptoms of this disease were very similar to those of brown rot caused by *Monilinia* spp. At a later stage, in mummified fruits, black pycnidia were formed on the surface of infected fruits under the skin. The pycnidia contained typical hyaline, nonseptate, fusiform, 15–29×5–8 μm conidia observed with a compound microscope. The pathogen was identified as *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips based on the ITS and EF sequences (GenBank no. JN135282). No mycelium and sporulation were observed on the infected mature fruits. Koch's postulates were satisfied after reisolating the fungus from artificially inoculated peaches.

Distribution of *N. parvum* in Imathia

In the first year of this investigation, *N. parvum* was only isolated from 30% of the total mature rotted peaches and 8% of the blighted shoots collected only from the location "Ammos-Mesi-Meliki", Veria, of the Prefecture of Imathia. In the following year, *N. parvum* was also found in other locations (Diavatos, Veria, Kopanos, and Agia Marina, all in the Prefecture of Imathia), isolated from both rotted fruits and blighted shoots, but at very low incidences (less than 3% and 5% of the total rotted peaches and blighted shoots, respectively).

Pycnidia of *N. parvum* were also found on blighted shoots and mummies retained on the trees.

Effect of temperature on mycelial growth and conidial germination of *N. parvum*

The optimum temperature for mycelial growth of *N. parvum* on PDA was 25°C, whereas mycelial growth was inhibited at 35°C and 2–4°C. The mycelial growth of *N. parvum* was statistically similar at 20 and 30°C, and significant higher than that at 15°C. Finally, the mycelial growth of the fungus at 15°C was significantly higher than 10°C. Regression analysis (Fig. 1) was used to describe the predicted mycelial growth of the fungus.

The results also showed that the rate of conidial germination and the germ tube elongation were reduced as temperatures increased from 25 to 30°C, decreased from 25 to 10°C, and was totally inhibited at 35 and 2–4°C. The percentage of conidia germination and the germ tube elongation at 15°C was signifantly higher than 10°C, but significantly less than 20°C. Regression analysis (Figs. 2 and 3) was used to describe the predicted percentages of conidia germination and germ tube elongation.

Pathogenicity and virulence of *N. parvum*

In inoculation tests, *N. parvum* was pathogenic to annual shoots of different peach and nectarine cultivars. No significant differences in virulence were observed among the isolates of *N. parvum* used. The cultivar Big Top was the most susceptible and the cultivar Maria Bianca the least susceptible as assessed by measuring the length of cankers (Table 1). The cultivars Sun Cloud, Sweet Gold, Symphony, Fantasia, O'Henry, Red Haven, Fayette, Tasty Free, Fire Blight, Red Gold, Venus, June Gold and Early Gold were significantly less susceptible than Big Top. The

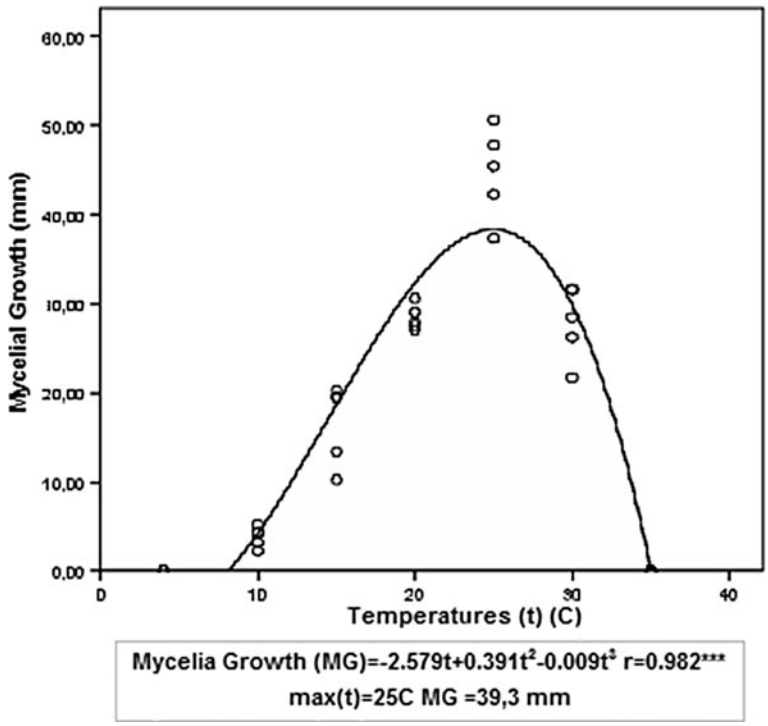

Fig. 1 Effect of temperatures on mycelial growth of the fungus *Neofusicoccum parvum*

cultivars Symphony, Menelaos, Rita Star, H. D. Hale, Caltesse 2000, Andriana, May Grand, First Gold, Crest Haven, Harmon and Sun Crest were not significantly different than Maria Bianca.

Pathogenicity and virulence of *N. parvum* and *D. seriata* isolates to peach fruit

All isolates of *N. parvum* and *D. seriata* used in inoculating peach fruit were pathogenic and resulted in similar sized rotted lesion (Table 2). Therefore, all these isolates were equally virulent to peach fruit.

Discussion

In this study the importance of the fungus *Neofusicoccum parvum* as the causal agent of a fruit rot

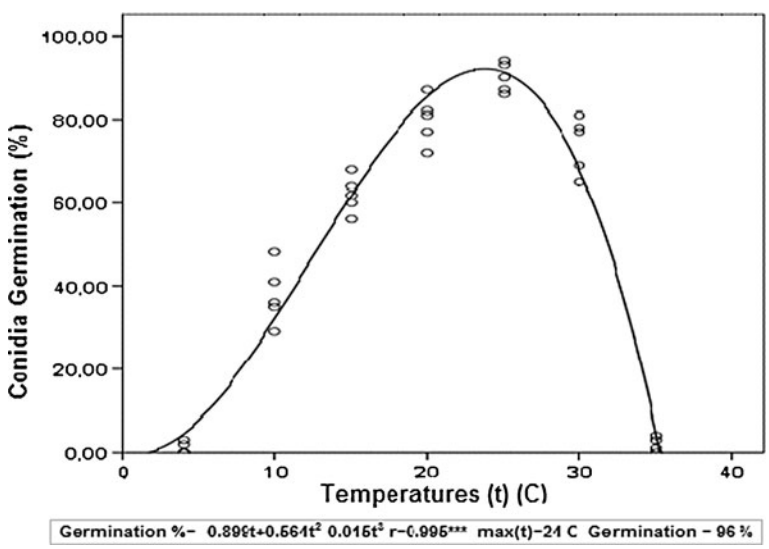

Fig. 2 Effect of temperatures on conidia germination of the fungus *Neofusicoccum parvum*

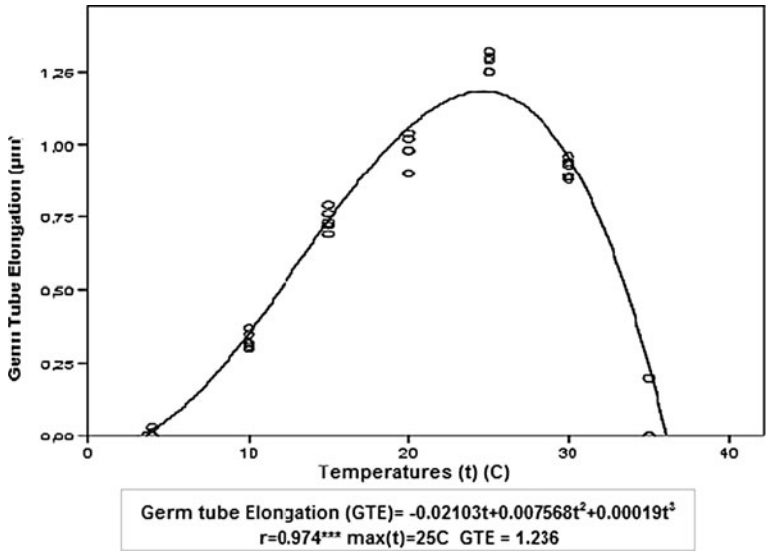

Fig. 3 Effect of temperatures on germ tube elongation of the fungus *Neofusicoccum parvum*

and a shoot blight diseases of peach in Greece was investigated. In previous works, *Botryosphaeria dothidea* was associated with fruit rots of peach in South Africa and the southeastern United States (Combrink et al. 1984; Brown and Britton 1986), while a *Dothiorella dothidea*, an asexual stage of a *Botryosphaeriaceae* species, was implicated as a cause of fruit rots of peach in Venezuella (Cedelo et al. (1994). The fungus *N. parvum* was isolated from blighted shoots of *Sequoiadendron giganteum* in Greece (Tsopelas et al. 2008) and pistachio in Greece (Inderbitzin et al. 2010). Also there was a report of *Botryosphaeria parva* causing shoot blight of kiwifruit in Greece (Rumbos and Phillips 2005). *Neofusicoccum vitifusiforme* and *N. australe* were associated with wood necrosis of plum, peach, nectarine and apricot in South Africa (Damm et al. 2007). In addition, *N. mediterraneum,* a species morphologically similar to *N. parvum*, was isolated from blighted shoots of *Eucalyptus* sp. on the island of Rhodos, Greece, and rotted olive fruit in Italy (Crous et al. 2007), Spain and the USA (Moral et al. 2010). These and our report indicate that *N. parvum* has a wide host range.

In this study, the fungus *N. parvum* was found overwintering as pycnidia on blighted shoots and mummified fruits (mummies) retained on trees. Studies with other species of Botryosphaeriaceae, such as *B. dothidea* causing panicle and shoot blight, showed that the pathogen overwinters on retained rachises, mummies, petioles, and cankers of blighted shoots (Michailides 1991). Following rains, conidia

Table 2 Pathogenicity and virulence of *Neofusicoccum parvum* from peach and *Diplodia seriata* isolates originated from other different hosts

Isolates	Original host of isolate	S.E.	Diameter of rotted area (cm)	
N. parvum	Peach	0.321	4.37[x]	a
D. seriata	Walnut	0.334	3.83	a
N. parvum	Peach	0.203	3.75	a
D. seriata	Permisson	0.237	3.60	a
D. seriata	Pistachio	0.231	3.44	a
D. seriata	Pistachio	0.270	3.43	a

[x] Values are the means of two experiments with 20 replicates each; values in the same column followed by different letters were significantly different at $P=0.05$ according to the Wald Test

released from pycnidia present on the previous year's blighted shoots, rachises, cankers, buds, and petioles of pistachio serve as the primary inoculum and cause the primary infections in the spring and early summer (Michailides 1991). Conidia from pycnidia formed on mummified peach fruit, endophytic infections, cankers on peach shoots formed the previous year and possibly pycnidia and ascocarps produced on other hosts in the neighbourhood of peach orchards can provide inoculum for primary infections, particularly since there are no other sources of *N. parvum* in the orchard. According to Amponsah et al. (2009) *Botryosphaeria* species, including *B. parva* from infected grapevines and other woody hosts, produce symptomatic infections on green grapevine shoots. New pycnidia that develop during the growing season in the summer and autumn serve as inoculum for secondary infections late in the season (Michailides 1991). Similarly, pycnidia that develop on green peach fruit and infectected shoots may provide inoculum for secondary infections of maturing fruit to cause fruit rots.

This study also showed that the optimum temperature for mycelial growth and conidia germination of *N. parvum* was 25ºC. Temperatures around 20–30ºC were common in the areas were the disease was problematic during the period of May–September. According to Ploetz et al. (2009) this fungus caused significant external symptoms, vascular discolouration, and mortality on *Syzygium paniculatum (*Myrtaceae) at 25 and 30°C; in general, only vascular symptoms developed at 20°C and no symptoms developed at 15°C. Similarly, Pitt et al. (2009) reported temperatures around 25ºC as optimum for infection of grapevine from *N. parvum.*

Although a comparison of susceptibility among the different cultivars used in the survey was not possible in the field—there were no peach/nectarine cultivars in Greece known to be resistant or susceptible (control) to *N. parvum*—all cultivars were susceptible and were infected. Relative differences in the level of susceptibility among the peach and the nectarine cultivars tested have been found. Beckman and Reilly (2005) tested the relative susceptibility of 25 peach cultivars to *B. dothidea* and found significant variation, with the cultivar 'Summergold' being the most susceptible and 'Redskin' the least. Diversity in the susceptibility of peach cultivars to *B. dothidea* was also reported by Okie and Pusey (1996) in Georgia, USA.

In testing various *N. parvum* isolates from peach, no significant differences in their virulence were found. Similarly, *D. seriata* isolates from persimmon and walnut infected peach equally well (Table 2). In contrast to our results, Amponsah et al. (2009) working with different members of Botryosphaeriaceae from various hosts (i.e. *B. parvum, B. lutea, B. australis, B. stevensii,* and *D. seriata*) found different virulence among the isolates on green grapevine shoots. Ma and Michailides (2002) found that isolates of *B. dothidea* from different hosts showed different levels of virulence on pistachio trees. Genetic variation among *B. dothidea* isolates from different host has been reported by Ma et al. (2001). However, more studies are needed, including a larger number of isolates of *N. parvum* from Greek peaches, before any definite conclusion on the virulence of this species can be made.

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